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INVESTIGATIONS OF REACTIONS OF POSSIBLE ANALYTICAL VALUE FOR URIC ACID

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BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Investigations of Reactions of Possible Analytical Value for Uric Acid" submitted by Ellenor Tomm Neumann in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

An investigation of the chemical reactions of uric acid was made in an attempt to develop a new, quantitative method for the estimation of serum uric acid. The method should be based on a reaction with uric acid which is more sensitive and specific than the phosphotungstic acid reduction reaction, and which would give results comparable in accuracy and precision to the spectrophotometric technique using the specific enzyme, uricase. The new method should be of routine analytical value and involve the use of instruments readily available to small hospital laboratories.

A study was undertaken to demonstrate that methods based on the reducing properties of uric acid such as the phosphotungstic acid reaction do not give results comparable in accuracy to those obtained using spectrophotometric techniques with uricase. A significant difference was obtained between determination by the Kern, Stransky and Archibald phosphotungstic acid method and a modification of the enzymic method of Praetorius. These studies show that the nonspecific colorimetric method does not reflect the true state of uric acid in serum and indicate the need for a new method for routine clinical use which is simple and precise.

The chemistry of uric acid and its degradation products was studied. Existing methods were reinvestigated to observe any fluorescent properties which may be inherent in the reaction products of the reactions investigated. New reactions and techniques were attempted in an effort to reveal

a reaction which could be measured colorimetrically or fluorometrically.

The most promising reagent investigated was 2,6-dichlorobenzoquinone chloroimide. This compound forms a colored reaction product with uric acid. Fluorescence characteristics were also observed. Unresolved difficulties were encountered with this substance in an effort to make the reaction of routine analytical value.

This reaction aroused interest in the behaviour of several aromatic compounds which would possess similar quinoneimine groupings upon oxidation. All attempts to produce a reaction with these compounds which would be suitable for the measurement of uric acid were unsuccessful.

The alkaline oxidation product of uric acid, allantoin, may be readily formed from uric acid in solution. Its quantitative determination was subjected to investigation. No useful reaction was developed.

Alloxan, the product of acid oxidation of uric acid, was also studied for the purpose of establishment of a new method for the quantitative estimation of uric acid. Although sensitive methods exist for the determination of alloxan fluorometrically, this laboratory was unable to report a suitable method for the quantitative oxidation of dilute solutions of uric acid to alloxan.

A clinical study of the variations in serum uric acid levels in one case of pernicious anemia was followed before and after treatment. The results of the study are in agreement with the results of Riddle, namely, that normal levels are found during relapse and an increase is observed starting one day after treatment.

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I. General Introduction

The determination of uric acid, the main end product of purine metabolism in man, is of considerable importance to the clinician in the diagnosis of certain disease states. The accuracy of these determinations is the problem of the clinical chemist.

The present study was undertaken to investigate the possibility of establishment of a method using colorimetric or fluorometric techniques. The new method should involve a more specific reaction for uric acid and should result in accuracy and precision superior to that available with present colorimetric methods.

The most widely used routine methods for the estimation of uric acid are based on its property of reducing phosphotungstic acid (1,2,3). Several modifications of the reaction have been made in an effort to increase the specificity of the reaction or the stability of the reaction (3-8). Poor recoveries and erratic standard curves are reported by many hospitals including the clinical laboratories of the University of Alberta Hospital. The University of Minnesota Hospital reports a rejection rate of 25% on all uric acid determinations (9). Even a cursory examination of medical literature reveals the inadequacies of nonspecific methods now routinely used (10). In spite of the inconsistencies encountered with

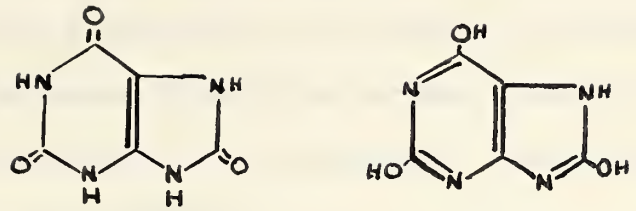
the phosphotungstic acid color reaction, very little research into other chromophoric or fluorophoric reactions has been attempted.

Much interest is now being directed toward the spectrophotometric technique which employs the specific enzyme, uricase, as reagent for uric acid determination (11-14). This method makes use of the strong absorption which uric acid exhibits in the ultraviolet region. However, even the uricase method is subject to inherent difficulties. A highly purified and uniform supply of uricase must be readily available. Also, because of the presence of proteins, it is necessary to measure relatively small changes in absorption of ultraviolet light in the presence of very high total absorption. By virtue of its specificity, the uricase method is certainly the method of choice but few hospitals and other laboratories will have the rather expensive ultraviolet instrument which is required.

For these reasons investigations of uric acid reactions seemed worthy in the light of the possibility of developing a new procedure suitable for smaller laboratories.

A. CHEMISTRY OF URIC ACID, ALLANTOIN AND ALLOXAN

Uric acid, a cyclic ureide, was discovered by Scheele in 1776 as a constituent of human urinary calculi.



keto form enol form

FIGURE 1

URIC ACID

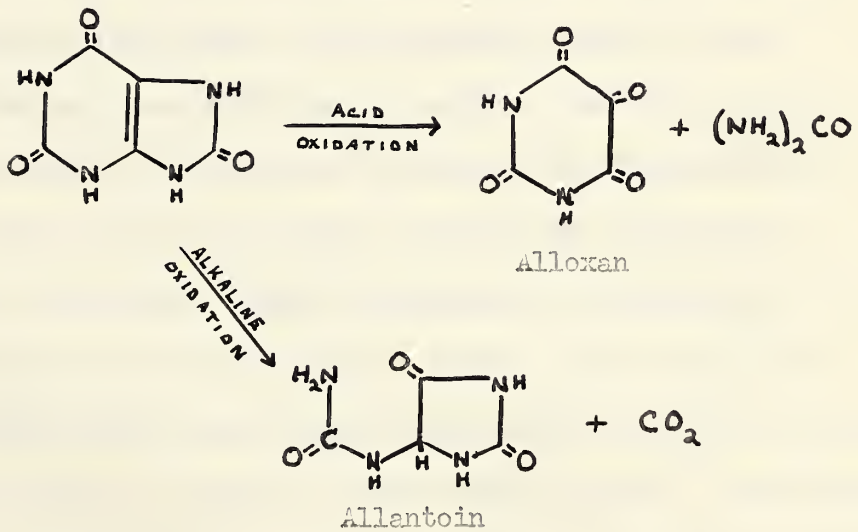


FIGURE 2

THE OXIDATION OF URIC ACID

The final elucidation and proof of structure was given by Emil Fischer in 1899. Uric acid belongs to the group of compounds having the same skeletal structure and referred to as the purine bases. According to systematic nomenclature of purines, it is the 2,6,8 trihydroxy purine. It is now believed that this purine exists in the amide form and accordingly its systematic name would be 2,6,8-purinetrione (see figure 1).

Uric acid is essentially a dibasic acid with ionization constants of pK_a 5.7 and pK_a 10.3. The protons on N atoms 3 and 9 are responsible for the ionization but which of the two is most readily removed is not known. The protons at position 1 and 7 may also be removed but only with great difficulty (15). Acid and neutral are the two classes of salts formed, with the acid urates being the most insoluble. The greater part of the sediment formed upon cooling urine is the acid urates. Crystalline uric acid may be formed by acidification of urine.

Uric acid is a white, odorless, tasteless crystalline substance which decomposes on heating. It is very insoluble in cold water, at 18°C 1 part uric acid dissolves in 39,480 parts water, and only sparingly soluble in boiling water, 1 part in 1,250 parts water. Solutions of uric acid deteriorate gradually when allowed to remain at room temperature and exposed to sunlight. Bacteria may elaborate the enzyme uricase which would rapidly

alter the concentration of standard solutions (3). Alcohol, chloroform, benzene, ether and other organic solvents do not dissolve uric acid, except certain organic bases such as ethylamine. It is soluble in concentrated sulfuric acid, in basic solutions and in alkali carbonates. Silver, mercuric, magnesium, ammonium, cuprous and cupric cations precipitate uric acid.

Most reducing agents do not affect uric acid but it is very susceptible to oxidizing agents (see figure 2). The products of oxidation obtained vary with the conditions employed although in general it is the pH of the medium rather than the nature of the oxidizing agent which is important.

The alkaline oxidation of uric acid can give allantoin, oxonic acid, allantoxaidin, oxaluric acid or other products according to the conditions (16). The course of the oxidation and even the structure of the products have been and still are fields of speculation.

Uricase, which acts as an aerobic dehydrogenase, catalyses the oxidation of uric acid to an unstable product which can be decomposed in two different ways without the assistance of enzymes. Both enzymic and alkaline permanganate oxidation yield the same end-product; the C_6 atom is lost, the attack taking place on the C_5 atom. The nitrogen atoms are equally distributed between the ureido group and the imidazole ring. Increasing pH accelerates the oxidation in both cases. Normally, a quantitative conversion of uric acid to allantoin

and carbon dioxide is obtained but in the presence of borate, there is also formation of other nitrogenous substances (17).

Oxidation of uric acid in acid solution gives mainly alloxan but prolonged contact with acid gives other products. Alloxan may be obtained by the careful oxidation of uric acid with nitric acid, chlorine (potassium chlorate and hydrochloric acid) or bromine.

The formation of a purple color due to the ammonium salt of purpuric acid (murexide reaction) when ammonia is added to the solution from nitric acid oxidation, is a characteristic reaction of solid uric acid and related purines.

The literature contains very little concerning reactions with uric acid other than aqueous reactions involving its reducing properties or formation of alkyl and chloro derivatives from solid uric acid. Two color reactions are recorded. A yellow color is formed in the presence of potassium persulfate when p-N-methyl amino phenol or amino phenol is added to an alkaline solution of uric acid (18). A red color is produced when a solution of 2,6 dichloro-benzoquinone chloroimide is added to an alkaline solution containing uric acid and silver ion subsequently added (19).

As mentioned, uric acid shows strong absorption in the ultraviolet. In the acid form, i.e. unionized form, the wavelength of maximum absorption is 285 mμ. As the urate salt, maximum absorption is observed from 290 - 295 mμ. The molar extinction coefficient of the urate at 292 mμ is 12,500. (See figure 3).

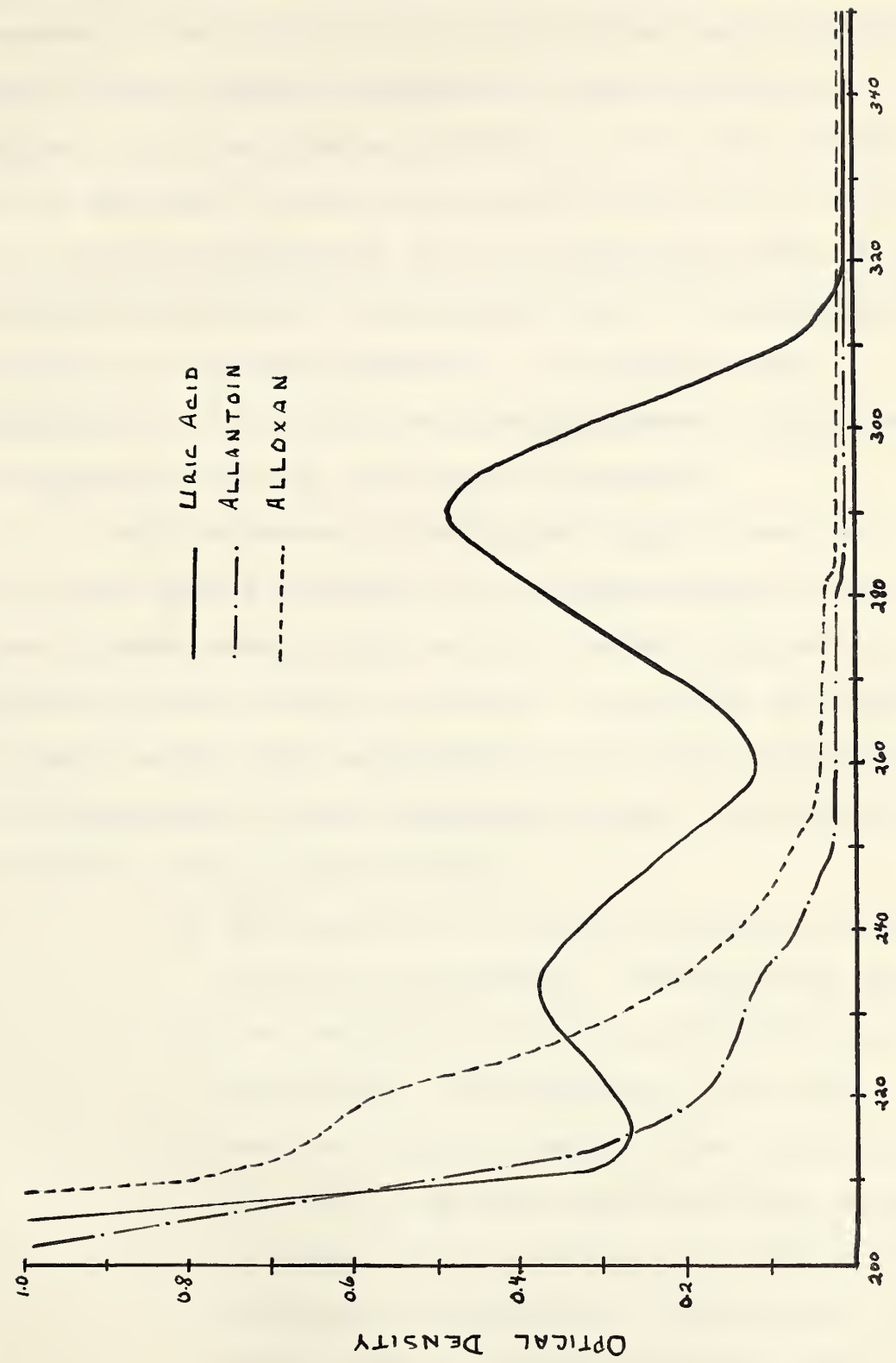


Figure 3

Absorption Spectra of Uric Acid, Allantoin and Alloxan

Another property of uric acid is its ability to fluoresce. When activated with ultraviolet energy of wavelength 325 mμ, radiant energy with a peak at 370 mμ is emitted. The fluorescence radiation is very weak and can only be detected in uric acid concentrations of 1 to 50 μg per ml. This fluorescence is of no analytical value as it requires an ultraviolet fluorometer which is not generally available in a routine laboratory. In addition, the concentration of uric acid required to observe fluorescence is relatively high for this type of procedure.

With reference to biological significance, uric acid is the main end-product of purine metabolism in man. In sub primate mammals, uric acid is further oxidized to allantoin by the action of uricase. In reptiles and birds, uric acid is the chief end-product of nitrogen metabolism and is comparable to urea excretion in man. The origins of uric acid in man are given below:

a) the breakdown of ingested preformed nucleic acids and nucleotides: the exogenous uric acid.

b) the breakdown of tissue nucleoproteins and nucleotides: the endogenous uric acid.

c) evidence exists to suggest that in man a small fraction of purines synthesized from glycine and other simple nitrogen and carbon-containing compounds is converted to uric acid by a more direct mechanism without the obligatory incorporation into nucleoproteins.

Whether the nucleic acids originate from foods or from tissue nucleoproteins, the pattern of their breakdown will be the same. The known breakdown pathways of adenylic and guanylic acid are given in the following figure.

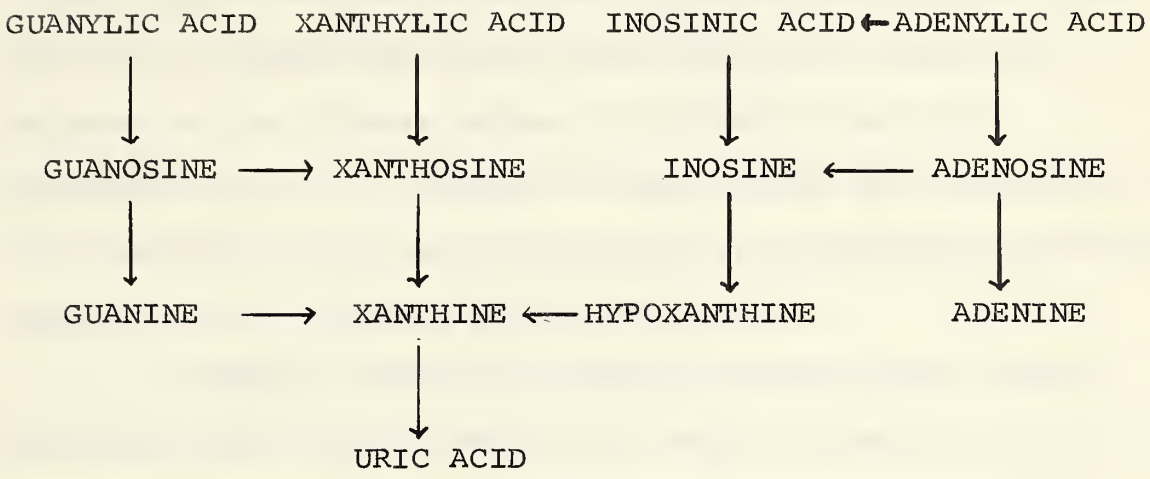


Figure 4

Breakdown of adenylic and guanylic acids in the body (Adapted from Sorenson (20))

Pathologically, deviations from normal levels may be of diagnostic value in certain disease conditions to the clinician. Gout and toxemia of pregnancy (21) are two abnormal conditions for which uric acid determinations are of such value. The implication of uric acid in gout is well established but the exact role it plays in the disease process is not completely understood. Attempts to associate an elevated serum uric acid with the cause of gouty attacks has come under considerable criticism since drugs affecting

the one may not influence the other (22,23). It has been suggested by Sorensen (20) that, "an elevated pool of uric acid can be regarded as a new biochemical criterion for the diagnosis of gout, a criterion which is far more accurate than an elevation of the concentration of uric acid in serum". In the future the determination of purine derivatives other than uric acid may be of more value when the biochemical pathways of the disease processes in gout have been elucidated (22,23). Probably a step toward that direction is the observation that increased excretion of 8-hydroxy-7-methyl guanine occurs during acute episodes (24).

Medical literature contains conflicting reports concerning the level of serum uric acid in patients with pernicous anemia (25,26,27).

B. REVIEW OF URIC ACID METHODOLOGY

Most methods for the determination of uric acid in solution are dependant upon the ease with which uric acid is oxidized in an alkaline medium. While uric acid is the substance oxidized, these methods are dependant on the presence of another substance capable of being measured in some manner, usually colorimetrically, upon its reduction by the uric acid. Phosphotungstic acid and arsenotungstic acid (1) are reduced with the formation of a blue color suitable for colorimetric estimation. Uric acid may also be determined by the color of iodine in chloroform after the reduction of iodic acid (28). Ferricyanide is reduced to ferrocyanide in alkaline solution (29).

The prussian blue formed gives a measure of the uric acid concentration.

To achieve more specificity, as the property of reduction is necessarily a nonspecific one, uric acid has been separated as an insoluble urate (3,4,30) and then determined with the colorimetric methods just indicated as well as by other oxidizing substances such as iodine, dichromate, permanganate and chloramine. Potentiometric methods have been used after oxidation with potassium ferricyanide (31). Uric acid and purine bases have been determined nephelometrically as silver salts in the presence of albumin (32). The spectrophotometric changes accompanying the oxidation of uric acid to allantoin and other end-products by uricase is the basis of another analytical method (11). Uric acid has even been estimated by the amount of urea formed after hydrolysis (33).

The most popular routine methods used are based on the reduction of phosphotungstic acid. The original method of Folin as modified by Brown (5), employs the use of sodium cyanide and urea to stabilize the color and prevent turbidity. The chemical nature of this effect is unknown. Kern, Stransky and Archibald (34) suggest the use of glycerine-silicate reagent and sodium polyanethol sulfonate to achieve the same purpose. Another modification makes use of sodium carbonate solution (35) as the ideal medium for color development and still another the combined use of sodium carbonate and lithium sulfate (36).

In spite of the many modifications, it still remains that the phosphotungstic acid reaction is nonspecific for uric acid. As a result, if the estimation is to have any significance, reducing substances other than uric acid must be absent or accounted for. Substances which interfere by reducing the reagent include: aromatic amines; hydroxyphenyl derivatives; aromatic aldehydes; unsaturated, hydroxy and keto acids; hydroxy methyl furfural and inulin; indoles; most of the morphine alkaloids; glutathione, ergothionine, cysteine, and cystine; glucose, ascorbic acid and ferrous salts. Most of the reducing sulfur containing compounds are present largely in the erythrocyte and are consequently eliminated since plasma or serum is used. The effect of phenols and glucose are negligible under the conditions of the reaction. Other of these interfering substances such as morphine alkaloids are not natural constituents of biological fluids. Amino acids are said to decrease the color as well as a certain substance present in the red cell (3). With interfering substances present it is difficult to evaluate the over-all qualitative and quantitative specificity of the method. Such factors as the proper medium and most favourable oxidation-reduction potential and also a medium for the most ideal development and stability of color become very important.

Another very serious source of error is the coprecipitation of uric acid by adsorption in the precipitation of the proteins. This constitutes a decrease of considerable

magnitude but can be minimized to some extent by slow and careful precipitation of the proteins. Shapiro (38) and other workers (39) suggest the partial isolation of uric acid from many of the interfering substances by the use of anion exchange columns. This also avoids coprecipitation. Any sulfur containing reducing substances which may be eluted along with uric acid are inactivated by the use of N-ethyl maleimide (39). The uric acid in the eluate may then be determined by any of a number of methods. Both spectrophotometric analysis and the phosphotungstic acid color reaction have been suggested after use of the ion exchange column.

The major difficulties encountered in routine laboratories with the phosphotungstic acid method are the unsatisfactory recoveries and the failure to obtain reproducible standard curves that follow Beer's Law. It is not uncommon to have recoveries of the order of 65% - 85%. At lower concentrations, a curve approximating a straight line is obtained. The curve rarely proceeds through the origin, i.e. zero absorption is not obtained for zero concentration. In order to achieve color development at a uniform rate, the time, temperature, and concentration of all reagents must be the same for each sample whether blank, recovery or test.

Every method for the estimation of uric acid has its own particular range of normal values. The same method may vary in response under different conditions and, therefore, it is advisable that laboratories establish their own normal range under the conditions they usually perform the test.

II. Experimental

The following results were obtained while attempting to develop either a color or fluorescent reaction with uric acid or its breakdown products, allantoin and alloxan. Aqueous standard solutions of uric acid with concentration ranging from 100 mg. per 100 ml. to 0.3 mg. per 100 ml. were used in the investigations. Reagents were dissolved in suitable solvents preferably water. Reactivity with uric acid, allantoin and alloxan was observed at several different pHs and in some cases under different oxidation conditions. Any special treatment used is indicated. In every instance the solutions were observed for color formation as well as the development of fluorescence. To observe for any possible fluorescence, two handlamps were used, one which emitted light in the near ultraviolet (350 - 450 mμ) and the other in the far ultraviolet (250 - 380 mμ). When a reaction was apparent, confirmation that uric acid was responsible for the reaction was made by absorption measurements at 293 mμ.

A. INVESTIGATIONS WITH URIC ACID

1. Precipitation Reactions

a) Inorganic Cations

Precipitation of uric acid is effected by mercuric, silver, ammonium, cupric, cuprous and lead cations. This property has been the basis of uric acid determinations by several workers. Bergmann and Dikstein (40) report a method suitable for routine clinical assays in which uric acid

is selectively precipitated as the mercuric salt in the presence of other purine derivatives. The precipitate is redissolved in sodium chloride and the optical density of uric acid measured at 290 mμ. Uzan (30) presents a similar method in which uric acid is isolated as the silver urate and the redissolved uric acid measured by potassium ferricyanide oxidation.

In an effort to establish a colorimetric method based upon the same principle, investigation into the measurement of mercuric cation precipitated was undertaken. Standard solutions of uric acid were precipitated using 0.1 M mercuric acetate. The precipitate was centrifuged, washed, and redissolved in 10% sodium chloride in 0.1 N hydrochloric acid. The amount of mercuric ion precipitated was determined by, i) the blue color formed with alcoholic diphenyl carbazone; ii) the orange color of the mercuric-dithizone complex in chloroform. The results obtained were inconsistent with expected values for concentration of uric acid in range 3 - 100 μgm. per ml. Repeated attempts gave similar results. When applied to serum protein free filtrates, the results were highly erratic. Mechanical loss of precipitate in the washing steps and the coprecipitation of mercuric cations are considered factors contributing to the discrepancies. The filtrate obtained after precipitation and centrifugation was analyzed in the ultraviolet for absorption at 290 mμ. In every case, a very small degree of absorption was observed. This would

indicate that the precipitate is slightly soluble under the conditions used and in the concentrations of uric acid present. The effect of pH on the precipitation was found to produce a change in the extent to which uric acid was being precipitated. The pH range of 1 to 6 was found to be the most favourable for more complete precipitation. Absorption measurements at 290 mμ indicate that a small fraction of uric acid remains in the filtrate after precipitation even under the most ideal conditions found. The wash solution contained no urate as shown by optical density examination.

b) Organometallo Precipitants

The review of literature revealed that phenyl mercuriborate (1:100,000 solution) (41) gives a yellow color with 3% solution of uric acid. This reaction was investigated for possible analytical value. In this laboratory, a color reaction with the reagent and uric acid was not obtained. Instead, every attempt to duplicate the reaction as recorded, resulted in the formation of a milky white gelatinous precipitate. The mercuric atom was in this instance responsible for the precipitation. The sticky precipitate was difficult to handle. After centrifugation, washing and hot hydrolysis in 1N hydrochloric acid, the mercuric atom released was determined using 1% alcoholic diphenyl carbazone. No correlation between amount of mercuric ion found and the uric acid content was observed. The filtrate and wash solutions showed trace amounts of soluble urate.

Acetoxy mercurianiline was also examined. Similar results were obtained.

c) Discussion

The advantage of the isolation of uric acid as the insoluble urate and the subsequent determination of uric acid after resolution is that interfering substances such as ascorbic acid, cysteine glutathione and amino acids will be absent. The methods using precipitation techniques described in the literature are satisfactory when applied to urine because of the greater concentration of uric acid and the larger volume available. However, in view of the appreciable solubility of the urate in such low concentration as is found in the blood (about 50 µgm. per ml. before protein precipitation), and the large volume required to make an accurate analysis, methods based on the precipitation properties are inferior to the reduction reaction of phosphotungstic acid as modified by Kern and Stransky. In addition, precipitation reactions are as nonspecific as reduction reactions. The estimation of the precipitating cation involves more steps in the procedure at which errors may occur. These disadvantages make such methods of even less routine analytical value.

2. Carbamyl Group Reactions

a) Diacetyl monoxime Reaction

Carbamyl compounds such as uric acid and its degradation products, allantoin and hydantoin, contain the

ureide group, R_1-NH-CO-NH-R_2. Ureide groups are susceptible to determination by diacetyl monoxime. The diacetyl monoxime reaction was carried out according to the procedure of Fearon (42) and also a modification by Koritz and Cohen (43). The latter method involves the intensification of the color if the reaction is carried out in the presence of an aromatic amine. In this case, sodium diphenylamine-p-sulfonate was used. The chromogenic equivalents on a molar bases for urea, uric acid, allantoin and hydantoin (taking the color produced by a 10 μmolar solution of urea as unity) are 1.0, 0, 0.4, 0.1 respectively. Absorption at 560 mμ was measured.

The interest in this urea reaction was not for color production but rather for the possibility of fluorescence characteristics of the reaction product by which it may be specifically determined. No fluorescence was observed after activation over the entire range of the spectrum from 250 to 780 mμ using the Aminco Bowman Spectrophotofluorometer.

b) Dimethyl amino benzaldehyde

Color reactions with dimethyl amino benzaldehyde and ureido acids, and methyl derivatives of uric acid are reported (44). The reaction was investigated for both color formation and fluorescence uric acid. Using the procedures described in the literature, neither color nor fluorescence was obtained with this reagent.

c) Discussion

It is suspected that the color produced by allantoin and hydantoin with diacetyl monoxime may have been

partly due to the formation of urea under the conditions of the reaction. The ureido groups present in the bicyclic structure of uric acid tend to make this compound relatively unreactive.

3. Reaction with Quinoneimine Groupings

a) 2,6-Dichlorobenzoquinone chloroimide (DCQC)

The phenol reagent, 2,6-dichlorobenzoquinone chloroimide gives a yellow color with uric acid which turns red upon the addition of silver ions (46). Theophylline and caffeine, similar in structure to uric acid, produce a blue color with this reagent (47). The reaction with uric acid has been postulated by Gibbs as in figure 5. A pH greater than 10 favours the deterioration of the reagent into brown condensation products and at pH less than 7 no reaction can be detected. The presence of large amounts of chloride ion contribute to the stability of the reagent in the reaction mixture according to Gibbs. A procedure is described for the estimation of urinary uric acid in a borate buffer of pH 10.

A study of the reaction of uric acid and this reagent was undertaken in an effort to establish a suitable method for serum uric acid determinations. It was found that a yellow colored complex is formed almost instantaneously and that the reagent starts to deteriorate in the reaction mixture about 25 minutes after addition of reagents. The color shows general absorption in region 400 - 470 mμ with a

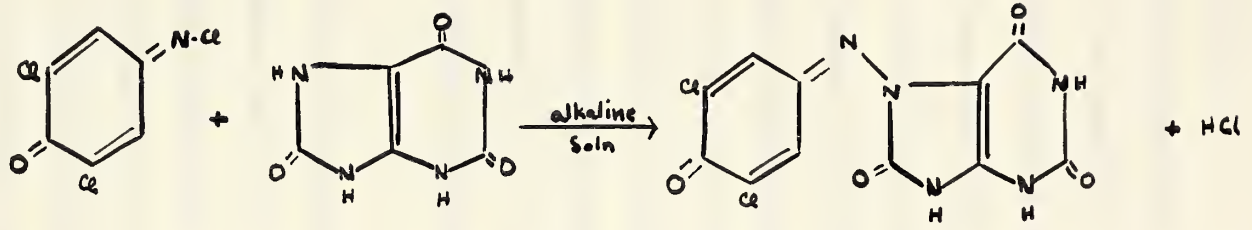


FIGURE 5

REACTION OF URIC ACID WITH 2,6-DICHLOROBENZOQUINONE CHLOROIMIDE. (GIBBS).

maximum between 430 - 440 mμ. Optical density measurements were of little analytical value since the brown deterioration pigments absorb more strongly at 430 - 440 mμ than the yellow colored reaction product.

i) Fluorescence Characteristics

The complex also exhibits a fluorescence having a maximum at 520 - 525 mμ when excited by wavelengths lying between 380 - 430 mμ. Maximum activation occurs at 410 mμ. Concentrations of uric acid down to 0.5 μgm. per ml. could be detected both by fluorescence and absorption measurements. A linear relationship between fluorescence intensity and uric acid concentration was obtained from 1 μgm. to 15 μgm. per ml.

The effect of a number of different buffers and pHs was observed. The most favourable conditions for the reaction were found using ammonium or borate buffers in the pH range 9 to 10. The procedure giving best results is as follows:

Test: 1 ml. uric acid solution (containing 1 - 30 μgm. per ml.)
1 ml. distilled water
2 ml. ammonium buffer pH 9.0
0.5 ml. 0.02% 2,6-dichlorobenzoquinone chloroimide (DCQC) in isopropyl alcohol
Measure fluorescence intensity within 15 minutes

Blank:

2 ml. distilled water

2 ml. buffer pH 9.0

0.5 ml. DCQC in isopropyl alcohol

Measure the fluorescence intensity within 15 minutes

It was observed that the reagent deteriorated more rapidly when used in amounts excessive to those indicated. The deterioration could be observed visually by the formation of brown pigments and fluorometrically by a fluorescence with maximum at 610 mμ when activated at 580 mμ. Increased pH also enhances this unfavourable effect. Under the conditions as described, the effect was rarely encountered before 30 minutes after initiation of the reaction.

The above results were obtained under the most sensitive response conditions of the Aminco Bowman Spectrophotofluorometer. The fluorescence of the reaction product is so weak that it is not at all detected by less sensitive instruments such as the Coleman Photofluorometer. Since the fluorescence yield of the product is so low, fluorometric techniques proved too insensitive for routine purposes.

ii) Extraction Attempts

Attempts were made to extract the colored substance into an organic phase in the hopes that an organic solvent would enhance the fluorescence. Dioxane, N,N dimethyl formamide, isobutanol, N butanol, ethyl ether, chloroform,

toluene, benzene, pyridine, o-xylene were all tried. No change was observed in either phase after repeated attempts at extraction. When a solution containing the reaction mixture was treated with ethanol, the formation of deteriorated reagent was enhanced. It appears then, that aqueous solutions favour the stability of the reagent and the colored reaction product.

iii) Attempts to Increase Sensitivity

The complexing of silver ion with the reaction product to form a red colored substance suggested that other cations may produce a similar effect without causing the undesirable chloride precipitation. 15 mono- and divalent cations were investigated. However, not one cation showed any useful complexing properties with the reaction product.

The usefulness of the uric acid - DCQC reaction as an analytical method seemed remote because of the low fluorescence yield and the nonspecific absorption characteristics. However, interest was aroused in other such quinone derivatives which may undergo similar quinoneimine reaction with uric acid.

b) <u>p-N-methyl amino phenol, amino phenol and benzoquinone chloroimine</u>

Uric acid forms a yellow colored product with these reagents in alkaline solution. In the case of the phenol derivatives, the presence of a suitable oxidizing agent such as potassium persulfate is necessary. p-amino phenols

readily undergo oxidation in alkali to form the benzoquinoneimine. The fluorescence and color produced in each case was investigated for analytical usefulness. Concentrations of uric acid detectable were not as low as those which could be detected by the 2,6 dichloro derivative. Fluorescence and absorption were observed at the same wavelengths as for DCQC but the magnitude of these characteristics was much lower.

Other chloro derivatives of benzoquinoneimide are not available. 2,6 dibromobenzoquinone bromoimide is available but gives the same results as the chloro counterpart.

c) 2,4-diamino phenol

2,4 diamino phenol was carefully oxidized in an alkaline medium in one case with potassium persulfate and in another with peroxide. Deep blue to purple colored solution resulted, indicating the formation of polyphenol dyes. Oxidized molecules of 2,4 diamino phenol reacted with species not oxidized to form the deeply colored pigments. Addition of uric acid to observe for a reaction was not attempted since any reaction which may have taken place could not be detected on account of the intense color of the reagent.

d) 1,8-diamino-4,5-dihydroxy anthroquinone

Solutions of this substance were blue in neutral and alkaline solution. Attempts to oxidize the reagent with oxidants potassium persulfate, potassium ferricyanide and hydrogen peroxide resulted in the formation of a pale yellow solution. Uric acid, when added to the

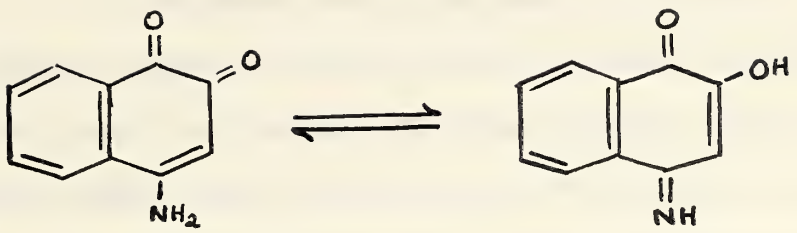
oxidized reagent and buffered at various pHs, produced no observable change. Absorption measurements indicated that the uric acid did not undergo reaction.

e) 4-amino-1,2-naphthoquinone

The reagent was prepared from 1,2-naphthoquinone by the procedure of Fieser and Hartwell[45]. The reagent is not available commercially. The product obtained also contained 2-amino-1,4 naphthoquinone, since the starting material was not obtainable in pure form and contained approximately 25% of 1,4 naphthoquinone. The two amino derivatives were not separatable by crystallization, consequently, the impure product was used for these studies. Very little information is contained in the literature concerning the formation of quinoneimines from aminoquinones of bi- and poly nuclear hydrocarbons. However, concerning 4-amino-1,2 naphthoquinone, Fieser and Fieser indicate that the hydroxy quinoneimine structure exists as the predominant tautomer in solutions of pH greater than 11.5. (See figure 6). The possibility that the hydroxy quinoneimine may give the same reaction as the benzoquinone seemed feasible. The hydroxy group at position 2 may obstruct any such reaction. The prepared reagent is red to brown in solution and is not changed even in strongly alkaline conditions. The addition of uric acid produced no visible or fluorometric change.

f) 4-amino-1-naphthol

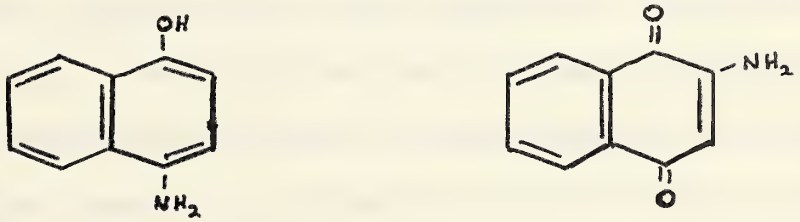
This substance was oxidized in a similar manner as the preceeding compounds. Repeated attempts to



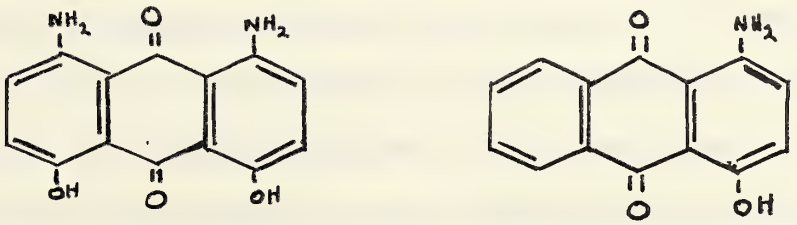
pH 0 - 11 pH 11 - 13

FIGURE 6

TAUTOMERISM OF 4-AMINO-1,2-NAPHTHOQUINONE



4-amino-1-naphthol 2-amino-1,4-naphthoquinone



1,8-diamino-4,5- dihydroxy anthroquinone

1-amino-4-hydroxy anthroquinone

FIGURE 7

SEVERAL COMPOUNDS INVESTIGATED

induce a reaction with uric acid were met by failure. Attempts with 2-amino-1,4-naphthoquinone proved as unsuccessful.

g) 1-amino-4-hydroxy anthroquinone

The above anthroquinone derivative is pale blue in neutral aqueous solutions and becomes yellow in the presence of alkali and oxidants. The reagent shows no fluorescence under these conditions. The addition of uric acid produces no change indicating that a reaction does not take place.

h) Discussion

Benzoquinoneimine is relatively unstable with hydrolysis to the quinone taking place readily. Chlorine and bromine substitution in position 2 and 6 appear to increase the stability and also affect the reactivity of the imide nitrogen. Amino hydroxy derivatives of naphthalene, anthracene and anthroquinone are more stable but whether the quinoneimine grouping is actually formed, except in the case of 4-amino-1,2-naphthoquinone, is not known.

The separation of the substance formed upon oxidation in alkaline medium was attempted. Since no reaction with uric acid was observed with the substance obtained after separation and crystallization, it is not known whether the quinoneimine which may have been originally formed was changed during the separation attempts or formed at all in the first place. There is no information in the literature to indicate that amino hydroxy derivatives of naphthalene, anthracene and anthroquinone may be converted to the quinoneimine form.

The reaction of uric acid in protein free filtrates with DCQC formed the characteristic color of the complex but the excess reagent and reaction product appeared stable for only a short time. Substances present in the filtrate increase the degradation of the reagent and obliterate fluorometric or colorimetric measurements. Even the use of slightly more than stoichiometric quantities of reagent did not reduce these effects to any great extent.

It is felt that the reaction between quinoneimine structures and uric acid may yield a useful analytical method but much more investigation into the chemistry of the reaction is imperative.

4. Miscellaneous Investigations

a) Fluorescence of Uric Acid

Uric acid, per se, fluoresces in the ultraviolet region in solution of pH 1. The fluorescence radiation emitted shows a peak at 370 mμ when activated maximumally by 325 mμ. (See figure 8). The fluorescence yield is low with only 1 μgm. per ml. detectable under most sensitive settings of the Aminco Bowman Spectrophotometer. Udenfriend, et al, claim an ultimate sensitivity of 0.7 μgm. per ml. with their instrument.

It was decided to investigate the possiblity of enhancing the fluorescence by employing different conditions. Only at very low pH does uric acid display fluorescence. A number of different acids at pH 1 were investigated with 0.1N

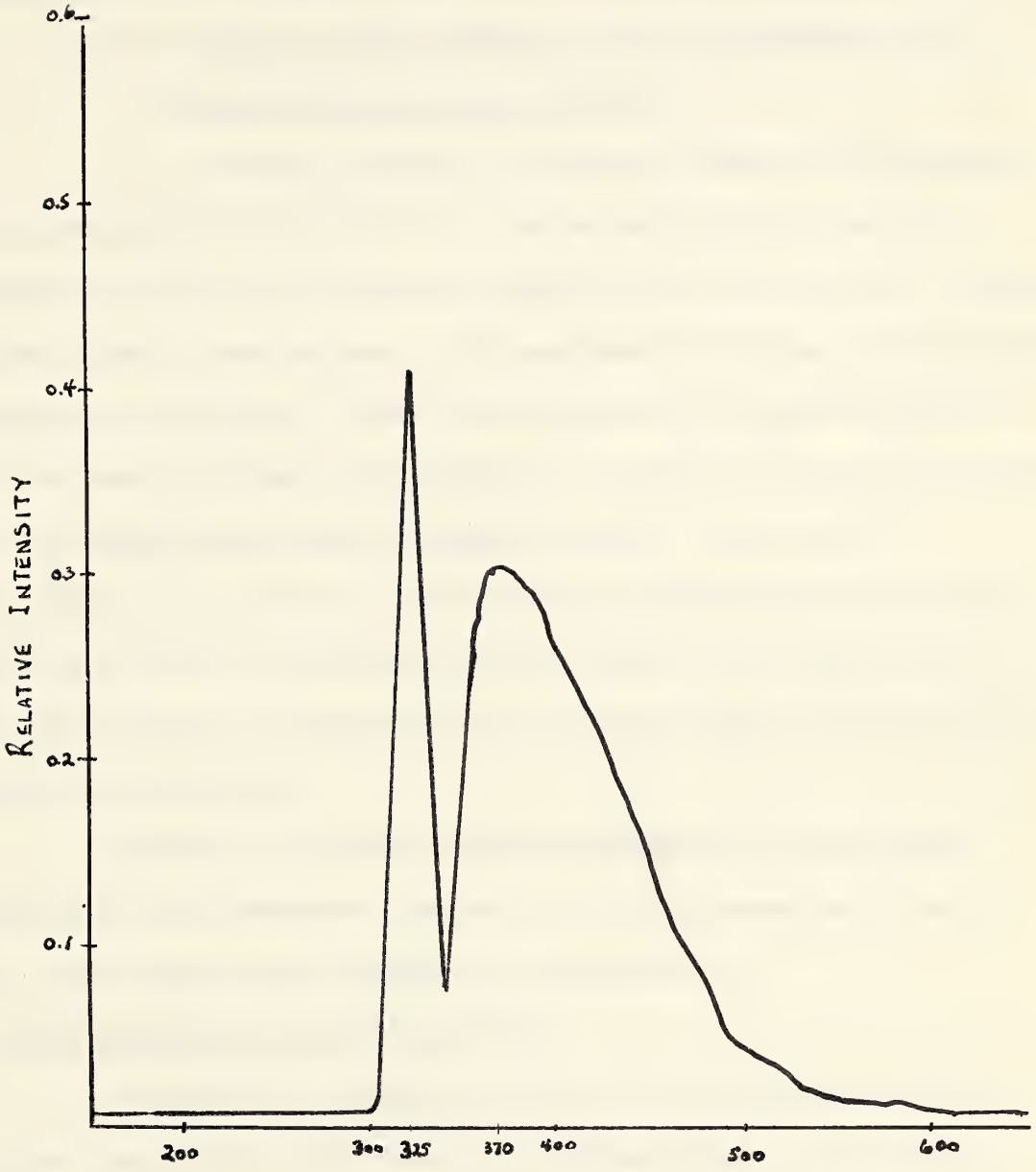


FIGURE 8

FLUORESCENCE SPECTRUM OF URIC ACID

Flu. max. 370 mμ

Act. max. 325 mμ

sulfuric acid, 0.1N nitric acid and 0.1N perchloric acid giving the best fluorescence yield. Increasing the acid strength did not increase the amount of fluorescence.

b) Effect of Uric Acid on the Fluorescence of Uranyl Acetate and Luminol

Uranyl acetate, 0.0001N, loses fluorescence in the presence of uric acid. The effect is not due to quenching but to the reducing properties of uric acid. Uranyl acetate shows fluorescence with maximum at 520 mμ. Activation maximum is at 315 mμ. When reduced, uranyl acetate shows no fluorescence at these wavelengths. For very dilute solutions of uric acid, i.e. below 20 μgm. per ml., the loss of fluorescence is linear. The foregoing observation is of no analytical value for blood analysis since the uranyl ion is very susceptible to quenching by a great number of ions most notably the halides.

Luminol, 3-amino-5-phthalhydrazide, also shows decrease in fluorescence intensity in the presence of uric acid. Here again the effect is nonspecific.

B. Investigations with Allantoin

Allantoin is readily obtained from uric acid by alkaline oxidation with potassium permanganate and by the catalytic action of uricase in alkaline buffers other than borate. It is a white crystalline substance fairly soluble in cold water and freely soluble in hot water. Phosphonium iodide and hydriodic acid oxidize it to hydantoin.

1. Methods for the Determination of Allantoin

Allantoin has been quantitatively determined after acid oxidation to glyoxylic acid which may be measured by condensation with 2,4-dinitro phenyl hydrazine (48). Another method in the literature makes use of ammoniacal copper and acid molybdate reagents (49). A large volume of sample is required for reproducible results in both of these methods. Each procedure was carried out to ascertain whether or not the reaction products possess fluorescence characteristics which may make the methods more sensitive. No fluorescence was observed in either case.

2. Potassium Bromide and Bromine

Another reaction by allantoin was also examined for possible fluorometric characteristics. This reaction involves the bromination of allantoin using potassium bromide and bromine in an alkaline solution (50). An unstable pink to violet color is obtained with concentrations above 100 µgm. per ml. No fluorescence is produced by the product.

3. Vanillin and Furfural

Aldehydes such as vanillin and furfural give a color with allantoin in the presence of hydrochloric acid. Attempts to repeat these reactions with dilute solutions of allantoin were unsuccessful.

4. Discussion

Solutions of allantoin were also added to the DCQC reagent concurrently with uric acid tests. Allantoin

does not react with this quinoneimine nor any of the other aromatic derivatives tried. It does give some color with diacetyl monoxime as shown previously but the reaction did not have any analytical value for dilute solutions of allantoin.

C. Investigations with Alloxan

Alloxan, the product of acid oxidation of uric acid, is the most reactive of the uric acid degradation products by virtue of the three adjacent keto groups in its structure. The substance is very soluble in water and in solution exists in the form of a stable mono hydrate. It may be obtained by the careful oxidation of solid uric acid with nitric acid, chlorine (potassium chlorate and hydrochloric acid), or bromine. According to work done in this laboratory, uric acid in dilute solutions (10 µgm. to 100 µgm. per ml.) is not quantitatively oxidized by these reagents since no alloxan was found by fluorometric methods and also since absorption of uric acid at 285 to 295 mµ was not reduced significantly. While the literature was being searched for an adequate method of acidic oxidations of dilute solutions of uric acid, reactions of alloxan were investigated.

1. Reaction with o-phenylene diamine

Alloxan may be quantitatively determined fluorometrically after condensation with o-phenylene diamine in acetate buffer (51). The method is very sensitive and excellent quantitative measurements are obtained in the range of 0.1 - 100 µgm. per ml. alloxan.

2. Determination as Riboflavin

In addition to the method cited, alloxan may be estimated as riboflavin after condensation with D-1-ribityl amino-2-amino-4,5-dimethyl benzene hydrochloride (52). The product had all the fluorescent properties of riboflavin but the reaction takes place slowly and is not quite quantitative. The former method was favoured.

3. Discussion

The literature revealed a procedure for the quantitative oxidation of uric acid to alloxan by vanadate ion in 6N sulfuric acid (53). When the reaction was attempted, it was found that the intense yellow color of the vanadate ion in 6N sulfuric acid changed to an intense brown to red color when the pH was changed to allow for optimum conditions for the reaction with o-phenylene diamine. Any fluorescent reaction which may have taken place was entirely obliterated by the vanadate color. It was concluded that the medium for oxidation of uric acid to alloxan by vanadate ion was useless for the subsequent fluorometric estimation of alloxan.

The literature was unsuccessfully searched for other methods of quantitative alloxan formation from solutions of uric acid. As a result, further study and investigation into the reactions of alloxan were abandoned.

III. Comparison of Three Methods for Estimation of Uric Acid

A. Introduction

Previous to investigations into the establishment of a new method, reinvestigation of the phosphotungstic acid

method as modified by Kern, Stransky and Archibald (34) (the procedure presently employed at the University of Alberta Hospital), and a comparison with the spectrophotometric enzyme technique was undertaken. Also included in the study was a modification of the colorimetric procedure which allowed for measurement of color "before" and "after" action of uricase. This particular modification of the preceeding method was included to observe whether the colorimetric procedure would show increased specificity for uric acid. The study should demonstrate how well the phosphotungstic acid method compares with the spectrophotometric uricase method.

B. Procedures

The reagents and procedure used for the Kern and Stransky method are given in Clin. Chem., 3, 102, 1957. (Archibald). In the second method which involved measurement "before" and "after" uricase treatment, buffer pH 9.3 was substituted for a portion of the water in the preceeding procedure. 25 µl of a concentrated preparation of uricase Leo was also added to both aliquots with one being allowed to stand 60 minutes at 37°C before the addition of precipitating reagent. The phosphotungstic acid used as precipitant stops the reaction. The third method compared was the enzymic method of Praetorius with procedure according to Liddle; J. Lab. Clin. Med. 54, 903, 1959. The reagents in the latter method were first calibrated to ensure that the reaction was proceeding correctly, i.e. to confirm the optical density factor of 0.0745 µgm. uric acid per ml.

The blue colored reaction product produced in the colorimetric methods was measured at 700 mμ at precisely 15 minutes after addition of the special uric acid reagent. The absorption spectrum of the colored reaction product is given in figure 9. Different concentrations of uric acid reach maximum color intensity at different rates. In spite of the lack of uniformity of color development, the change takes place at about the same rate in the unknown and standards. Figure 10 shows the rate of color development of two standard solutions using the Kern, Stransky and Archibald procedure.

C) Results

Refer to Table 1.

D) Discussion

The results of this study demonstrates that a significant difference exists between results obtained by the colorimetric and spectrophotometric uricase methods. This finding is in contradiction with the comparison studies of Kanabrocki, et al, (54) and also by Alper and Seitchik (55). It is supported in studies conducted by Feichtmeir and Wren (12) and Lous and Sylvest (56). No agreement exists as to which method gives the lower values.

In comparing the two colorimetric methods used, it was noted that instead of an expected lower mean, the colorimetric procedure employing uricase gave a slightly higher mean than the regular procedure of Kern, Stransky and Archibald. The slight elevation is due to a slight augmentation

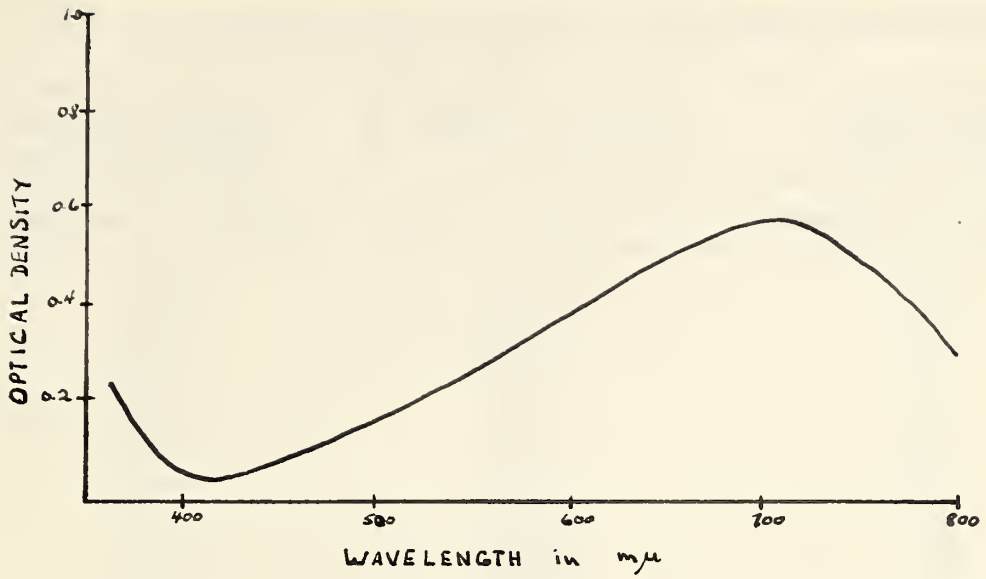
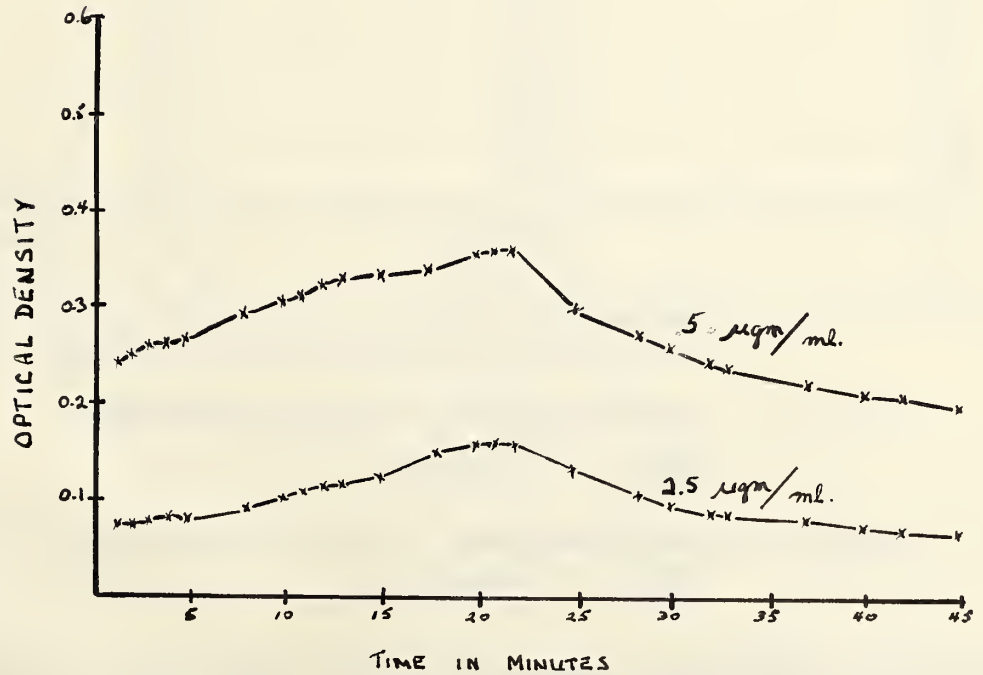


FIGURE 9

ABSORPTION SPECTRUM OF COLORED SOLUTION OBTAINED UPON PHOSPHOTUNGSTIC ACID REDUCTION



COLOR DEVELOPMENT OF 2 STANDARD SOLUTIONS WITH TIME

FIGURE 10

TABLE I

Results Obtained in Comparing Three Methods for the Determination of Uric Acid using Pooled Sera

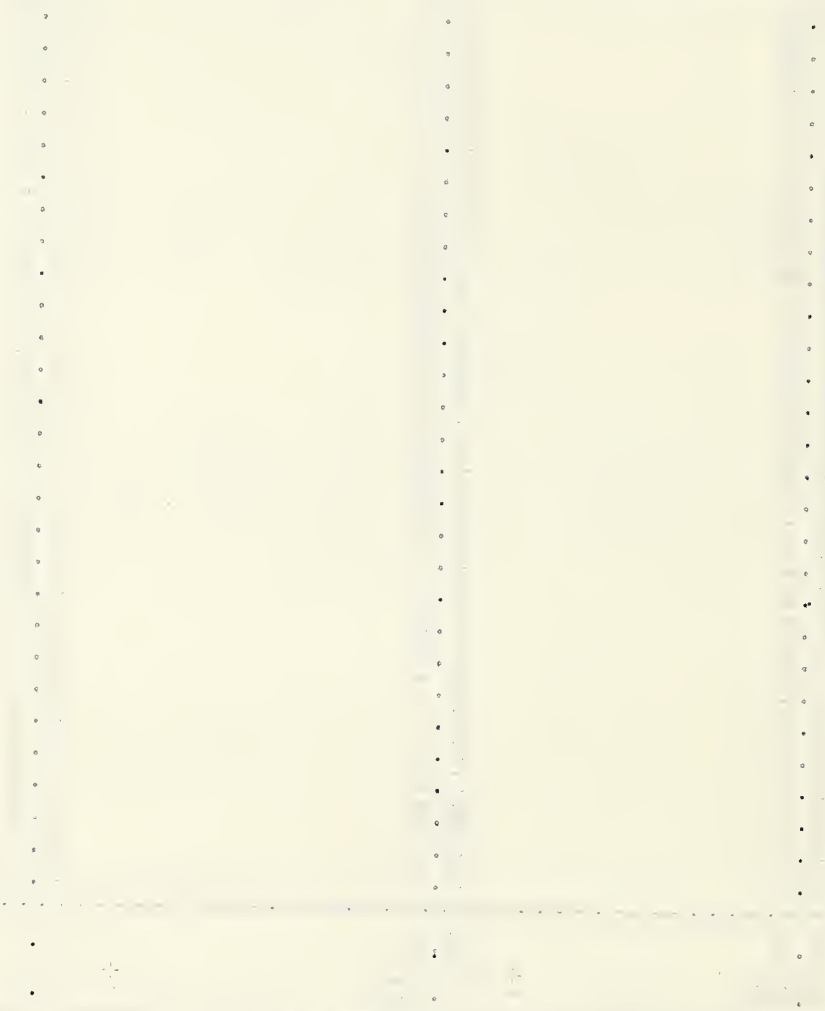
A. Kern, Stransky and Archibald	B. Enzyme modific. of A	C. Spectrophotometric method
mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
4.8	5.1	4.9
4.8	4.9	5.0
4.7	5.4	5.0
4.8	5.1	4.7
4.7	5.1	4.6
4.8	5.1	4.8
4.6	4.8	4.7
4.7	5.2	4.7
5.0	5.0	4.6
5.4	5.5	4.8
5.1	4.9	4.2
4.9	5.1	4.6
5.5	5.4	4.2
5.0	5.2	4.2
5.1	5.6	4.7
5.0	4.9	4.7
5.7	5.2	4.6
5.5	5.4	5.0
5.6	5.2	5.0
5.7	5.5	4.6
4.9	4.6	4.8
4.8	5.1	5.0
4.8	5.1	5.0
5.2	5.1	4.7
5.6	5.5	4.6
5.5	6.4	4.8
5.7	5.2	5.0
5.2	5.3	5.0
MEAN 5.1	5.2	4.7
S.D. $\pm$ 0.36	$\pm$ 0.33	$\pm$ 0.24

"t" test for significant differences:

Comparing A and B: $$\frac{5.2 - 5.1}{\sqrt{\left(\frac{.36}{28}\right)^2 + \left(\frac{.33}{28}\right)^2}} = 1.111$$

Comparing A and C: $$\frac{5.1 - 4.7}{\sqrt{\left(\frac{.36}{28}\right)^2 + \left(\frac{.24}{28}\right)^2}} = 4.666$$

A "t" value above 2.052 indicates significant difference at 95% levels.



of color by the buffer employed. Standard curves prepared using the same amount of buffer in the standards as in the test solutions showed a slight increase in slope when compared to curves obtained from solutions prepared without glycine. The test of significance shows that there is no significant difference between the results obtained by the two procedures.

IV. A Clinical Study of Serum Uric Acid Concentration in Pernicious Anemia

A. Introduction

A clinical study of the fluctuations in serum uric acid concentration was undertaken in a male patient with a relapse in pernicious anemia admitted to the University Hospital. The patient had a record of the disease for 12 years. Peripheral blood smear findings were very suggestive of pernicious anemia. Diagnosis was confirmed by the response to subsequent vitamin B_{12} therapy.

The purpose of the investigation was to establish the serum uric acid level during relapse and the direction of variation upon remission. The literature contains divergent findings (25,26,27) in studies made several decades ago using nonspecific methods. No recent information concerning uric acid in pernicious anemia was found.

B. Procedure and Results

The modification given by Liddle (13) of the spectrophotometric method using uricase was used in this study. Normal values for this method are 2.6 - 7.5 mgm. per 100 ml. for males.

Figure 11 shows the results obtained.

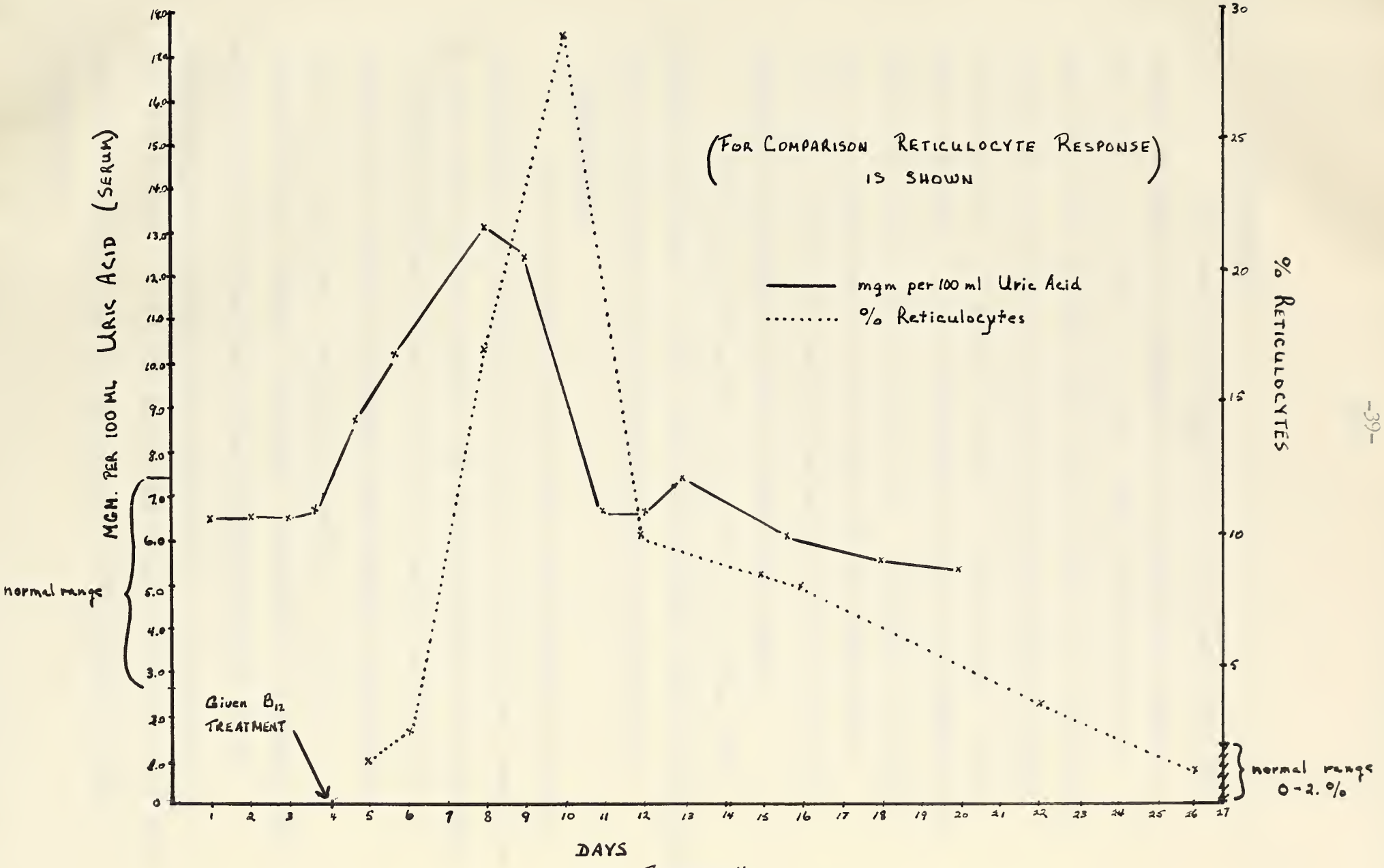


FIGURE II

VARIATION IN SERUM URIC ACID BEFORE AND DURING VIT. B_{12} THERAPY IN PERNICIOUS ANEMIA.

C. Discussion

The results obtained are in agreement with the results of Riddle who observed an increase above normal as soon as one day after treatment. The level returns to normal at about the same rate the red cells do. The results of this single study also agree with those found by Riddle during relapse. He observed normal or below normal values before treatment. This observation is not in agreement with studies by Gettler and Lindeman (27). A survey of a large number of cases must be made before one can establish a conclusive correlation between serum uric acid levels and changes in the disease state of pernicious anemia.

The elevated serum uric acid levels found after treatment may well be the result of increased nuclear metabolism which is associated with the maturation of erythrocytes.

V. Conclusions

The reluctance of uric acid and allantoin to undergo reaction with the formation of colored or fluorescent complexes by which they may be assayed, may be attributed to the presence of carbamyl groups in the structure. However, a reaction between uric acid and 2,6-dichlorobenzoquinone chloroimide does occur and forms a measurable product. It is too insensitive for routine techniques. Attempts to increase the sensitivity by extraction of the reaction product or by complexing with cations were unsuccessful. It is possible that this type of reaction may be of analytical value routinely but investigation into the chemistry of the reaction itself is important.

Future improvement of present methods will probably involve a simple quantitative technique for the isolation of uric acid before determination by means of its reducing properties. The estimation of allantoin after oxidation of uric acid with uricase is another direction of future investigation. Studies performed in this laboratory with allantoin were not successful in developing such a method. The lack of a quantitative method for the conversion of uric acid to alloxan is an obstacle to research from this approach.

The comparison study demonstrates the need for a method superior to the Kern, Stransky and Archibald method, and suitable for routine work in every clinical laboratory. This conclusion is based on the finding of a significant difference between results obtained by the procedure cited above and the specific spectrophotometric enzyme technique. The latter method, although the method of choice, is not employed in many laboratories since it necessitates the use of instruments not readily available in every laboratory.

The divergent findings in serum uric acid levels in pernicious anemia and the general lack of knowledge concerning disturbances in purine metabolism in other diseases suggest the need for a rapid, accurate method for uric acid. With such a method, a survey of large numbers of cases for variation in concentration and the correlation between variation and disease states could be made.

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